ELSEVIER

Journal of Chromatography A, 736 (1996) 97–104

JOURNAL OF CHROMATOGRAPHY A

Improved potency assay for recombinant bovine somatotropin by high-performance size-exclusion chromatography

Jen P. Chang[a,*], Thomas H. Ferguson[a], Paul A Record[b], David A. Dickson[a], Douglas E. Kiehl[a], Allison S. Kennington[a]

[a]*Lilly Research Laboratories, Eli Lilly and Co., Greenfield, IN 46140, USA*

[b]*Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285, USA*

Received 1 August 1995; revised 13 November 1995; accepted 20 November 1995

Abstract

A high-performance size-exclusion chromatographic method was developed for the determination of potency of recombinant bovine somatotropin (rbST) monomer and the estimation of dimer and soluble aggregates in bulk drug substances. These proteins can be completely extracted from bulk drug substances with sodium borate–ethylenediaminetetraacetic acid disodium salt (EDTA) at pH 9.5 and separated on TSK G3000SW column with a mobile phase of pH 7.3 sodium borate–EDTA. The results demonstrated that this method was a non-denaturing assay for the determination of potency of rbST monomer and the data obtained in this study correlated well with data of the hypophysectomized rat body weight gain bioassay. The rbST monomer and dimer in the separation were verified by liquid chromatography–electrospray mass spectrometry. This method was optimized and validated.

Keywords: Somatotropin; Recombinant proteins; Proteins

1. Introduction

Recombinant bovine somatotropin (rbST) has been used for increased milk production in lactating dairy cows. rbST is known to form dimer and high-molecular-mass oligomeric species, which are bio-inactive, in the isolation, purification, and formulation processes and upon storage [1–3]. It is necessary to have a reliable and accurate assay that can be used not only for the determination of bio-active monomer but also for the oligomers and aggregates. Traditionally, hypophysectomized rat bioassay [4,5] has been used for the determination of potency of growth hormone but this pseudo-quantitative method cannot discriminate between monomer, dimer, and aggregates. This assay is imprecise, costly and time-consuming. Recently, physicochemical techniques have been attempted as a replacement for the traditional biological assays, such as hypophysectomized rat bioassay [4,5] for the potency determination of growth hormone in routine analysis [6,7]. The important aspects to consider for the use of physicochemical methods for the determination of potency are that the methods should be non-denaturing and the results obtained should be correlated with the hypophysectomized rat bioassay. The physicochemical assay should be able to separate and quantify bio-inactive oligomers and aggregates which cannot be discriminated in the hypophysectomized rat bioassay.

*Corresponding author.

0021-9673/96/$15.00
SSDI 0021-9673(95)01284-2

High-performance size-exclusion chromatography (HPSEC) has been employed to determine the potency for recombinant growth hormone [6–8] because of the mild conditions in the chromatographic process and good efficiency in separation by molecular size. In the previous paper, we reported a non-denaturing potency assay for the determination of rbST in bulk materials by HPSEC [8]. In the method, a polymer base column and pH 9 ammonium hydrogencarbonate mobile phase were used for the HPSEC because rbST needs a weak alkali medium to maintain bioactivity. This method can be used for the determination of major bioactive species and the estimation of bio-inactive aggregates. A deficiency of the method was that the rbST dimer, if present, could not be separated from the monomer due to the poor separating resolution provided by the polymer based packing materials. In addition, the pH variability of the ammonium hydrogencarbonate mobile phase influenced reproducibility of analyses.

This paper describes an improved method for the determination of rbST potency in which a silica-based SEC column, TSK G3000 SW, and sodium borate–ethylenediaminetetraacetate (EDTA) buffer solution were used. This method appears to provide better resolution in the separation of the monomer, dimer and soluble aggregates and can be used for the measurement of the biopotency of slightly degraded preparations of rbST and estimation of dimer and soluble aggregates. Based upon the limitation of SEC, rbST degradation products such as deamidated, oxidized and other variants that have similar molecular size to rbST cannot be separated by this method. However, we have confirmed deamidated rbST monomer to be fully bio-active. Liquid chromatography–electrospray mass spectrometry was used to verify molecular masses of rbST monomer and dimer in the separation. In addition, the effects of pH and buffer concentration on the separation were studied.

2. Experimental

2.1. Materials

Recombinant bovine somatotropin bulk drug substances (Lot 010, Lot 011 and Lot 012) and reference standard (RS 0096) were obtained from Eli Lilly (Greenfield, IN, USA). Reagent-grade water was obtained from a Millipore Milli-Q water system. All other chemicals used were of analytical reagent grade or better.

2.2. HPLC

HPSEC was performed on a HPLC system consisting of a Waters 625 LC system with a 991+ photodiode-array detector and WISP 712 autosampler (Waters Chromatography, Milford, MA, USA). Columns evaluated in the development of this method included TSK G3000SW, TSK G3000SW$_{XL}$, TSK G2000SW$_{XL}$, 300×7.5 mm, TSK G3000SW 600×7.5 mm (TosoHasso, Montgomeryville, PA, USA), Shodex KW 802.5 protein column, 300×8 mm (Showa Denko, Tokyo, Japan), Synchropak GPC 100 column, 300×7.8 mm (Synchrom, Lafayette, IN, USA), Zorbax GF 250, 250×10 mm (MAC-MOD Analytical, Chadds Ford, PA, USA). A TSK G3000SW column 300×21.5 mm was used for the semi-preparative work with rbST dimer. All analytical columns were operated at ambient temperature and with a 20-μl injection volume. A flow-rate of 0.5 ml/min was employed for most of the studies. The mobile phase was 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution adjusted to pH 7.3 with hydrochloric acid, except where specified. Eluates were detected at 280 nm. A linear regression plot of the reference standard in mg/ml vs. peak area was used to quantify rbST monomer in samples. Peak-area normalization was employed for the estimation of dimer and aggregates. Chromatographic data were collected, stored and analyzed by a HP-1000 computer system (Hewlett-Packard, San Fernando, CA, USA).

2.3. Sample and reference standard preparation

A 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution adjusted pH to 9.5 with sodium hydroxide was used as a sample solvent to dissolve bulk drug substances and reference standard. Samples were prepared at a concentration range between 0.3 and 0.8 mg/ml for analyses. In order to minimize incomplete dissolution, an aliquot of the buffer

solution was added to the bulk drug substance and allowed to stand at room temperature for 40 min. The solution was then gently shaken for 5 min until completely dissolved and then diluted to volume.

2.4. Isolation of dimer

A 10 mg/ml concentration of rbST bulk drug substance was prepared in the sample solution. An aliquot of 400 μl of this solution was injected onto the semi-preparative HPLC system consisting of a TSK G3000 SW semi-preparative column and the same mobile phase used for analytical purposes. The isolation was carried out at a 2.5 ml/min flow-rate. The dimer fractions were collected and dialyzed against 5 m*M* ethylenediamine for 24 h. The dimer solution was then lyophilized.

2.5. Reversed-phase (RP) HPLC–electrospray mass spectrometry (ESP-MS)

A gradient elution with trifluoroacetic acid (TFA)–acetonitrile (ACN) solvent system was used in RP-HPLC–ESP-MS for the molecular mass measurement of rbST monomer and dimer. Solvent A was 0.1% TFA in 35% ACN–water and solvent B was 0.1% TFA in 70% ACN–water (70:30). A 30-min linear gradient from 0 to 100% solvent B at 1 ml/min flow-rate was used to separate samples on a Vydac 218TP104 column. The LC–MS experiment was conducted on a Sciex API LC–MS system (Perkin-Elmer/Sciex, Toronto, Canada). The split ratio was constant at 99.5 to 0.5 with 0.5% of the total post-column effluent introduced into the ESP-MS interface in the ESP-MS system. The other 99.5% passed through the flow cell of a Waters 486 tunable absorbance detector and the signal was monitored simultaneously at 214 nm with the acquisition of MS data.

Deconvolution of charged states and assignment of total molecular mass was achieved with the aid of the Hypermass feature included with the MacSpec mass spectral data manipulation package (Sciex). Marker ions detected in the assigned scan range were used to extrapolate mass–charge relationship to determine accurate mass for the components studied.

3. Results and discussion

3.1. HPSEC column

Several kinds of SEC columns were employed in this study to evaluate the column performance including resolution, reproducibility and column-to-column variability. A typical SEC chromatogram obtained on a TSK G3000 SW column is shown in Fig. 1. This column achieved the best reproducibility and chromatographic resolution in the separation of the three target species, rbST monomer, dimer and aggregates. TSK G3000SW$_{XL}$ achieved good resolution in the separation owing to the smaller particle size of packing material but the column-to-column variability and decreased column life-time limited this column for routine analysis. A longer TSK G3000SW column, 600×7.5 mm, showed excellent resolution in the separation of rbST monomer and dimer but aggregates were lost due to decomposition resulting from the longer chromatographic process in the column. Similar results were obtained on the Zorbax GF 250 column. Theoretically, TSK G2000SW$_{XL}$ should have better resolution in the separation but poor performance was found with this column. Shodex KW 802.5 and Synchropak GPC 100 achieved poor resolution of the monomer and dimer. As a result, the TSK G3000SW column was selected for the use in this method.

3.2. pH effect in solutions

pH effects on the solubility of rbST have been studied [1,8]. Results indicated that the rate of aggregation/precipitation increased with decreasing solution pH. In this study, we found that pH in the range of 6.7 to 11 in sodium borate–EDTA buffer solutions did not affect the elution time of the three targets in the HPSEC separation but strongly affected the peak areas of dimer, and particularly, the aggregates. The pH-dependent peak areas of rbST monomer, dimer and aggregates are presented in Fig. 2. We noted that the peak areas of dimer and aggregates were not dependent on solution pH at pH>9, but dramatically decreased with decreasing pH below 9. No dimer and aggregates peaks were found at pH below 7. At pH>10.5, however, rbST monomer was not stable and more dimer resulted in solution.

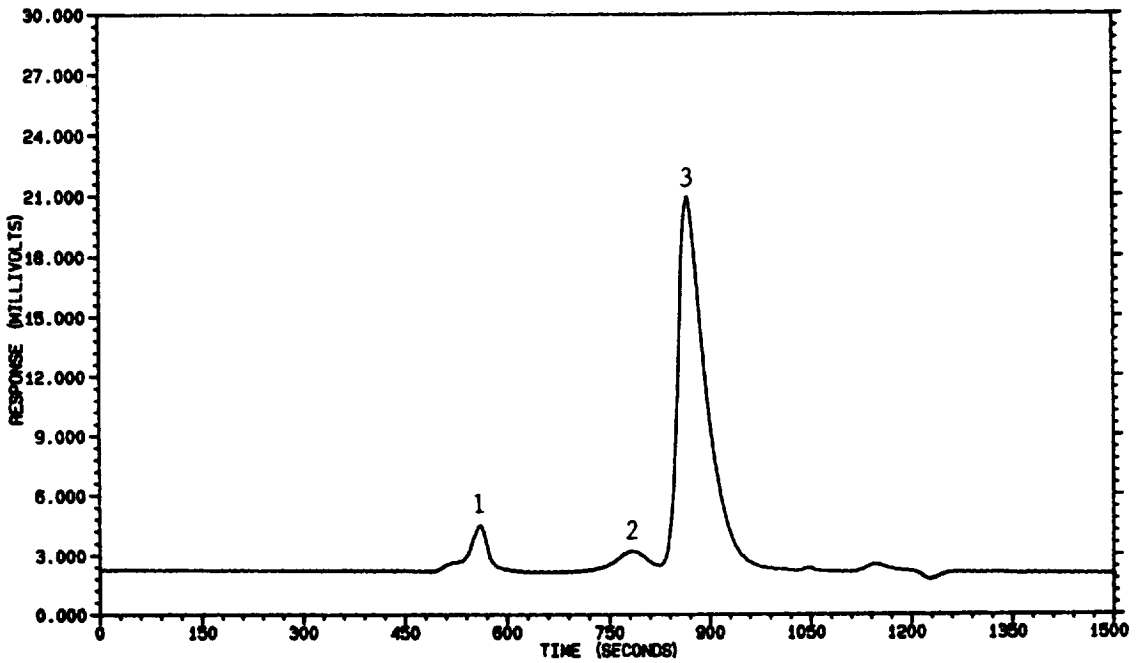

Fig. 1. HPSEC chromatogram of rbST, Lot 012. Chromatography was performed on a TSK G3000SW column and isocratically eluted by a mobile phase with 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution pH 7.3 at 0.5 ml/min flow-rate. Elutes were detected at 280 nm. Peak 1=aggregates; peak 2=dimer; peak 3=monomer.

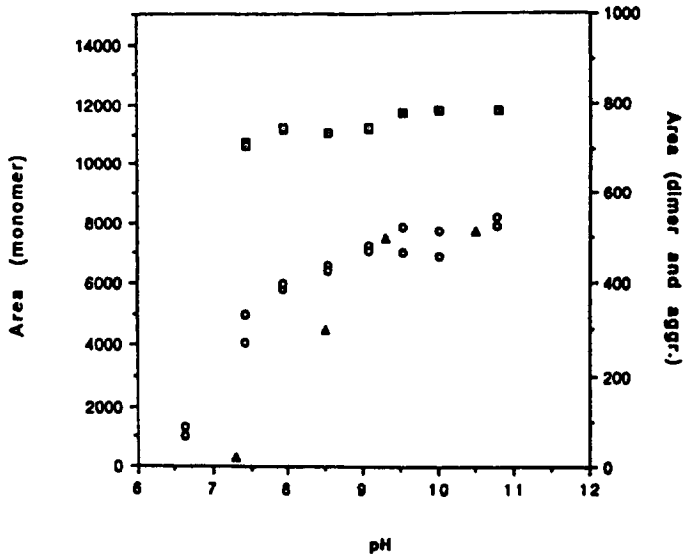

Fig. 2. Effect of pH on sample peak areas. The sample used in this experiment was rbST (Lot 012) and dissolved in 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution adjusted to different pHs with NaOH or HCl. The HPLC conditions were the same as in Fig. 1. (□) monomer; (○) dimer; (△) aggregates.

Therefore, the optimal condition for sample solution was pH 9.5 in sodium borate–EDTA buffer solution. In addition, the use of sodium borate–EDTA buffer with the same concentration as the mobile phase eliminates the influence of void peaks in the separation.

3.3. Mobile phase

In the previous paper [8], we indicated that rbST aggregates were strongly affected by pH and buffer concentration in ammonium hydrogencarbonate solutions because of the strong hydrophobicity of aggregates. Aggregates can only be present in higher pH (>9) buffer solution. However, the silica-based columns allowed pH<7.4 mobile phase to be used. In this study, a sodium borate–EDTA buffer at pH 7.3 was used in the separation and quantification of rbST and its oligomers. The pH of this buffer appears to be more stable than the ammonium hydrogencarbonate buffer. Consequently, rbST has excellent stability in this buffer solution. Second, this buffer demonstrates greater extraction efficiency when used in the extraction of rbST in formulated samples [9]. The effect of the buffer concentration in the mobile phase on peak areas of three target species is shown in Fig. 3. The results demonstrated that there was no significant effect of buffer concentration on peak areas of rbST monomer and dimer, but a strong effect was observed on the peak area of aggregates. When buffer concentration increased to 50 m*M* borate in the mobile phase, almost no aggregate peaks were found in the separation. Therefore, the soluble aggregates of rbST cannot be present in solutions with high buffer concentrations and low pH (see Fig. 2). The elution times of monomer and dimer increased with increasing buffer concentrations in the mobile phase (Fig. 4). A slight change in elution time of both the monomer and dimer was observed as the borate concentration increased to greater than 20 m*M*. The elution time of aggregates remained the same with increasing buffer concentration. EDTA present in the buffer solution enhances stability of rbST. On the basis of results obtained, 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution at pH 7.3 was used as the mobile phase in this method.

The effect of flow-rate was investigated. Variation

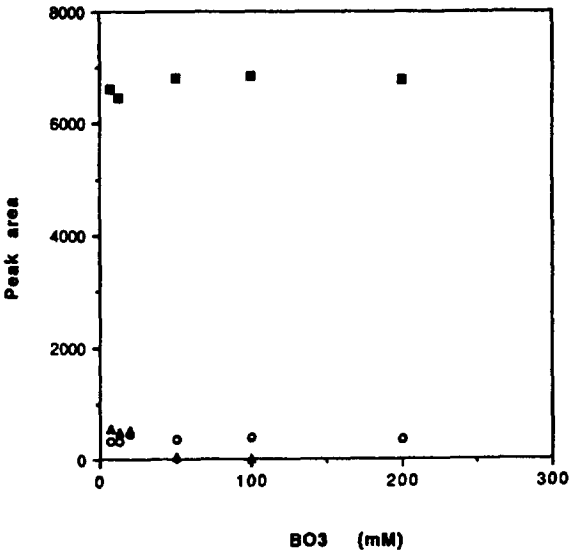

Fig. 3. Effect of borate concentration in the mobile phase on peak areas. The rbST (Lot 012) was dissolved in pH 9.5 20 m*M* sodium borate–1.44 m*M* EDTA buffer solution and chromatographed at the same conditions as in Fig. 1. (□) monomer; (○) dimer; (△) aggregates.

of flow-rate in the range of 0.25–1.0 ml/min did not affect the normalized peak-area ratio between the monomer, dimer, and soluble aggregates. The optional flow-rate in this method was 0.5 ml/min. A

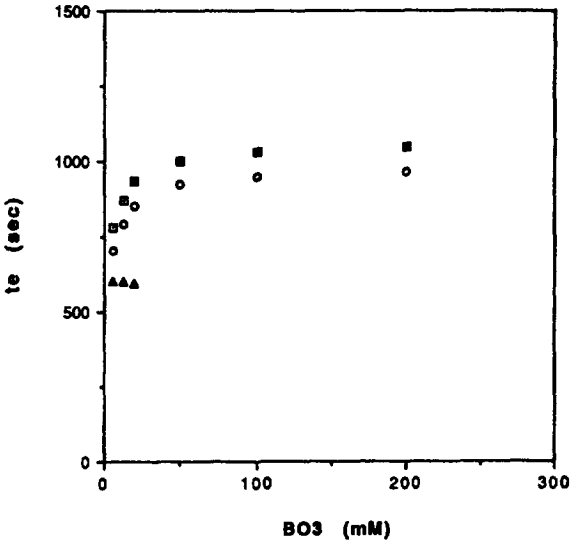

Fig. 4. Effect of borate concentration in the mobile phase on elution times. Experimental conditions were the same as in Fig. 3.

higher flow-rate of 1.0 ml/min resulted in the rbST main peak being partially overlapped with the dimer peak. No dissociation of the soluble aggregates was observed at a flow-rate of 0.25 ml/min.

3.4. Molecular mass measurements

The apparent molecular masses of rbST monomer and dimer have been confirmed by using RP-HPLC–ESP-MS. Results in Fig. 5 revealed that the bulk drug substance contained two components in the TFA–ACN mobile phase as evidenced by the ultraviolet (UV) and total ion chromatogram (TIC). Mass spectral data associated with the peak 1 eluting at around 22.5 min indicated that this component was rbST monomer with a molecular mass of 22 819 Da (calculated mass 22 818 Da). The smaller peak 2 at a retention time of approx. 25 min was suspected rbST dimer. This component was found to exhibit a mass of 45 638 Da by mass spectral data. No data was obtained for the molecular mass of rbST aggregates because they were dissociated by the strong acidic medium in the TFA–ACN mobile phase in the RP-HPLC. The isolated dimer component obtained by HPSEC is an enriched dimer mixture with the monomer. It provided further confirmatory evidence of the dimer identification. Fig. 6 shows comparative UV and TIC signal traces for this component. Spectral data obtained indicated that the first main peak represented rbST monomer with a mass of 22 819 Da. The second main peak was confirmed to be the rbST dimer with a mass of 45 638 Da. Two small peaks 3 and 4 in the profile have not been

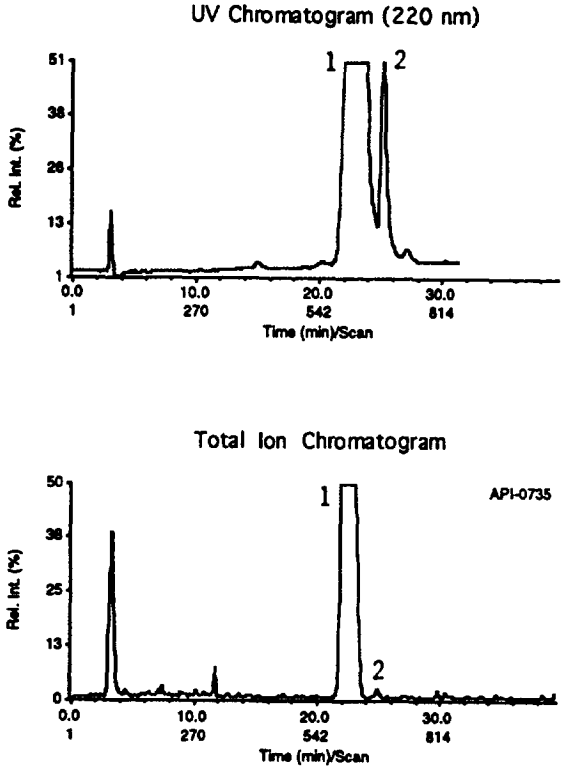

Fig. 5. UV chromatogram and total ion chromatogram of rbST (Lot 012) by RP-HPLC–MS. The reversed-phase HPLC was performed on a Vydac 214TP104 column equilibrated with 0.1% TFA in 35% ACN (A solvent) and eluted with a linear gradient from A solvent to B solvent (0.1% TFA in 70% ACN) within 30 min at flow-rate 1.0 ml/min. Other experimental conditions have been described in the Experimental section. Peak 1=monomeric rbST; peak 2=dimer.

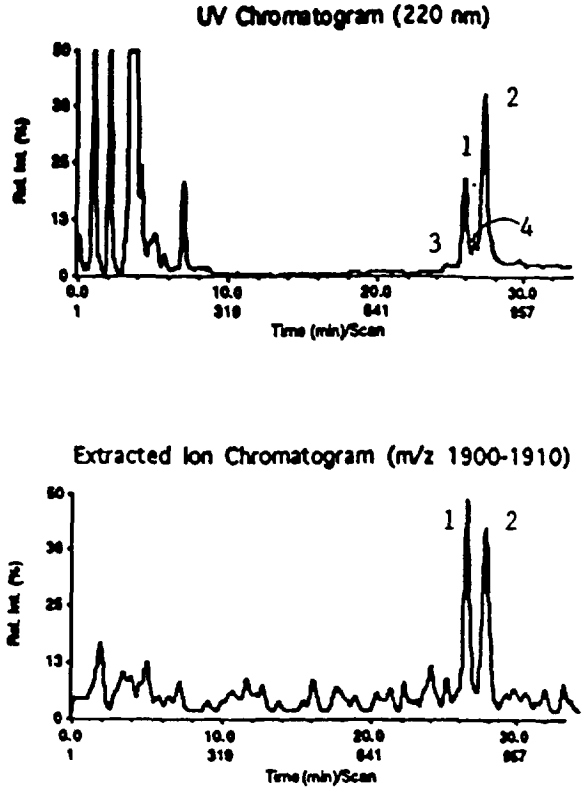

Fig. 6. UV chromatogram and extracted ion chromatogram of isolated rbST dimer collected fraction by RO-HPLC–MS. Experimental conditions were the same as in Fig. 5. Peak 1=rbST monomer; peak 2=rbST dimer; peaks 3 and 4=unknown.

identified yet because they are too low in concentration for RP-HPLC–ESP-MS measurement.

3.5. Correlation between rat body weight gain assay and HPSEC assay

The correlation between hypophysectomized rat body weight gain potency and HPSEC potency obtained by this method was investigated. Linear regression analysis of the data obtained by both assays using several lots of bulk drug substances is shown in Fig. 7. A regression coefficient of 0.899 was obtained. These results clearly demonstrate that the data obtained with this non-denaturing HPSEC method provides better precision and accuracy for rbST potency compared to hypophysectomized rat bioassay and it may be used to replace the hypophysectomized rat bioassay as a measure of potency assay for routine analyses.

3.6. Validation

The linearity of rbST monomer was evaluated by preparing different concentrations of rbST reference standard and the bulk drug substance from 0.05 to 2.0 mg/ml. A correlation coefficient of 0.999 was obtained for the monomer using linear regression analysis. The limit of quantification was 0.1 mg/ml.

Due to the difficulty of preparing the pure reference standard for both the dimer and aggregates, a normalization procedure with peak-area ratio was employed to estimate rbST dimer and aggregates in bulk drug substances. The linearity of dimer and aggregates was evaluated by using solutions with concentrations varying from 0.1 to 2.0 mg/ml (Fig. 8). The correlation coefficients were 0.999 for the lot containing 6% of dimer and 0.995 for the same lot containing 7% of aggregates. The limitation of estimation was 1%.

The precision of the method for determination of monomer and estimation of dimer and aggregates was assessed through triplicate analysis of three bulk samples for three different days. Results shown in Table 1 indicated that the intra-day precision for monomer was 3% and inter-day precision within three days was 1.24%. The precision of dimer and aggregates in inter-day study were less than 6% R.S.D. and about 20% R.S.D., respectively. Poor precision in the estimation of soluble aggregates was

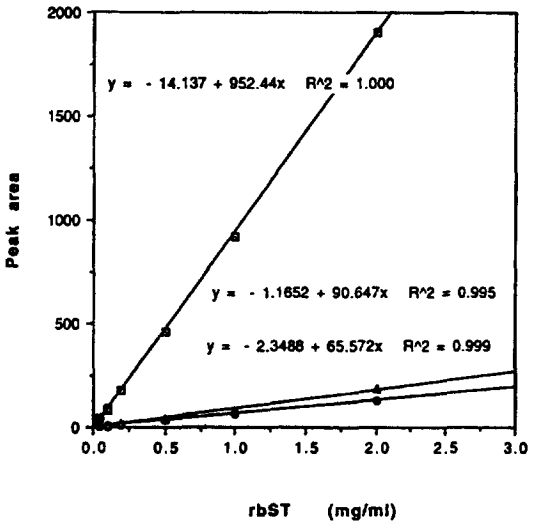

Fig. 7. Correlation between hypophysectomized rat body weight gain assay and improved non-denaturing HPSEC assay for rbST bulk drug substances.

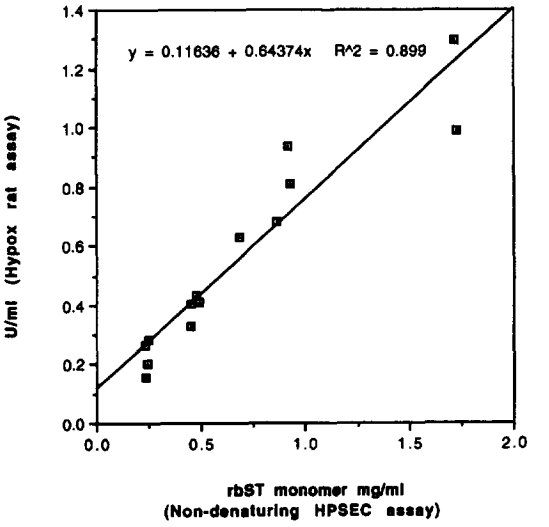

Fig. 8. The plots of linearity for rbST monomer, dimer, and aggregates. The regression coefficient over the dynamic range (0.05–2.0 mg/ml) for the monomer was 1.000, for the dimer it was 0.999 and for the aggregates the was 0.995. The experimental conditions used were the same as in Fig. 1. (□) monomer; (○) dimer; (△) aggregates.

Table 1
Precision (in %) of non-denaturing assay for bulk materials

	Lot 010			Lot 011			Lot 012		
	M	D	A	M	D	A	M	D	A
Day 1	93.78	2.51	0.89	91.28	2.14	1.01	82.37	5.67	8.24
R.S.D. (%) (n=3)	2.10	8.0	14.0	0.45	11.9	23.4	1.34	3.5	3.4
Day 2	92.77	2.54	0.63	91.94	2.27	0.79	81.21	5.48	8.70
R.S.D. (%) (n=3)	3.29	8.8	16.6	0.75	3.9	13.0	1.08	3.2	1.5
Day 3	95.10	2.50	0.84	92.83	2.25	0.86	81.65	5.78	7.97
R.S.D. (%) (n=5)	1.35	8.7	21.4	0.84	6.6	12.30	1.11	1.9	1.6
3 Days mean	93.89	2.51	0.79	92.01	2.22	0.89	81.65	5.64	8.30
R.S.D. (%)	2.32	4.7	19.4	0.95	5.9	16.2	1.11	3.0	4.2
Day-to-day	93.88	2.52	0.79	92.01	2.22	0.89	81.65	5.64	8.30
R.S.D. (%)	1.24	0.8	17.5	0.85	3.4	12.4	0.76	2.7	4.4

M=monomer; D=dimer; A=aggregates.

due to low content (<1%) in the bulk drug substances.

In order to assess the accuracy of this method, the solutions of two different lots were fortified with two different concentration of standard solution. The average recovery was 94.0% (R.S.D. 2.6%).

The stability of rbST in pH 9.5 20 m*M* sodium borate–1.44 m MEDTA buffer solutions was evaluated at 2–8°C and room temperature over a period of two days by three lots of bulk drug substances. The variability was found to be 2.5% for the monomer in sodium borate–EDTA solutions within two days. Degradation and oligomerization were found at pH> 10.5 buffer solution after two days. This result was consistent with that reported in the literature [2,3].

Acknowledgments

The authors thank Drs. Eugene L. Inman, Mark R. Coleman and Thomas K. Jeffers for their help with the review of the paper and Mrs. Priscilla Phelps for the preparation of the paper.

References

[1] M.J. Hageman, J.M. Bauer, P.L. Possert and R.T. Darrington, J. Agric. Food Chem., 40 (1992) 348.
[2] A.G. Severia, G.K. Strohl, O.F. Fox and K.S. Iyer, J. Agric. Food Chem., 40 (1992) 337.
[3] B.N. Violand, M. Takano, D.F. Curran and L.A. Bentle, J. Protein Chem., 8 (1989) 619.
[4] W. Marx, M.E. Simpson and H.M. Evans, Endocrinology, 30 (1942) 1.
[5] W. Evans, M.E. Simpson, W. Marx and E. Kirbrick, J. Endocrinol., 32 (1943) 13.
[6] A.F. Bristow and S.L. Jeffcoate, Phareuropa, 3 (1991) 3.
[7] R.M. Riggin, C.J. Shaarl, G.K. Dorulla, D.S. Lefeber and D.J. Miner, J. Chromatogr., 435 (1988) 307.
[8] J.P. Chang, R.C. Tucker, B.F. Ghrist and M.R. Coleman, J. Chromatogr. A, 675 (1994) 113.
[9] J.P. Chang, T.H. Ferguson, P.A. Record, D.A. Dickson and H.G. Richey, J. Liq. Chromatogr., submitted.